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Novel Quantitative Biosystem for Modeling Physiological Fluid Shear Stress on Cells[▽]

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The response of microbes to changes in the mechanical force of fluid shear has important implications for pathogens, which experience wide fluctuations in fluid shear in vivo during infection. However, the majority of studies have not cultured microbes under physiological fluid shear conditions within a range commonly encountered by microbes during host-pathogen interactions. Here we describe a convenient batch culture biosystem in which (i) the levels of fluid shear force can be varied within physiologically relevant ranges and quantified via mathematical models and (ii) large numbers of cells can be planktonically grown and harvested to examine the effect of fluid shear levels on microbial genomic and phenotypic responses. A quantitative model based on numerical simulations and in situ imaging analysis was developed to calculate the fluid shear imparted by spherical beads of different sizes on bacterial cell cultures grown in a rotating wall vessel (RWV) bioreactor. To demonstrate the application of this model, we subjected cultures of the bacterial pathogen *Salmonella enterica* serovar Typhimurium to three physiologically-relevant fluid shear ranges during growth in the RVW and demonstrated a progressive relationship between the applied fluid shear and the bacterial genetic and phenotypic responses. By applying this model to different cell types, including other bacterial pathogens, entire classes of genes and proteins involved in cellular interactions may be discovered that have not previously been identified during growth under conventional culture conditions, leading to new targets for vaccine and therapeutic development.

Pathogenic bacteria experience wide fluctuations in fluid shear levels during the natural course of infection, ranging from 4 to 50 dynes/cm^2 along blood vessel walls (7) to less than 1 dyne/cm^2 in utero and between the brush border microvilli of epithelial cells (1, 5, 6, 15, 24). While fluid shear has been reported to affect bacterial gene expression, physiology, and pathogenesis (3, 4, 19, 25, 26, 31, 32), the mechanism(s) by which this mechanical stimulation affects the response of bacterial pathogens has not been elucidated and has not been considered in the vast majority of studies. The principal limitation in the study of microbial response to fluid shear forces has been the lack of a model system to quantify variations in the fluid shear experienced during growth of planktonic (suspended) microbial cultures. Understanding multiscale biophysical phenomena, such as the mechanism behind the fluid shear response of cells, requires a multidisciplinary approach that incorporates the use of mathematical modeling to relate biological effects at large length scales to those at the cellular and subcellular levels. A rigorous combination of numerical modeling and well-characterized experimental microbial systems holds the potential to enhance our knowledge of bacterial pathogenesis and may lead to the identification of novel targets for vaccine and therapeutic development.

We report the development of a novel model system wherein we mathematically modeled the fluid shear within a modified rotating wall vessel (RWV) bioreactor and evaluated the response of planktonic cultures of *Salmonella enterica* serovar Typhimurium to quantified ranges of physiological fluid shear. The design of the RWV bioreactor permits cell growth in suspension culture (9, 17, 20, 32) and minimizes the fluid shear levels encountered by cells. The RWV is a rotating bioreactor in which cells are maintained in suspension in a gentle fluid orbit that creates a sustained low-fluid-shear environment for cell growth (Fig. 1A) (16, 20). The principal design of these reactors is based upon a cylindrical culture vessel, which is completely filled with medium (i.e., all bubbles are removed to reduce shear) and creates a solid body rotation as the vessel is rotated on its axis that is parallel to the ground. The solid body rotation of the media allows the organism to remain suspended at a constant terminal velocity and offsets the sedimentation of the bacteria in the reactor (13, 16, 27, 28). Under these culture conditions, the cells are maintained in suspension in a gentle fluid orbit as the RWV is rotated and a sustained low-fluid-shear environment for cell growth is achieved (Fig. 1B). A gas-permeable membrane on one side of the RWV allows

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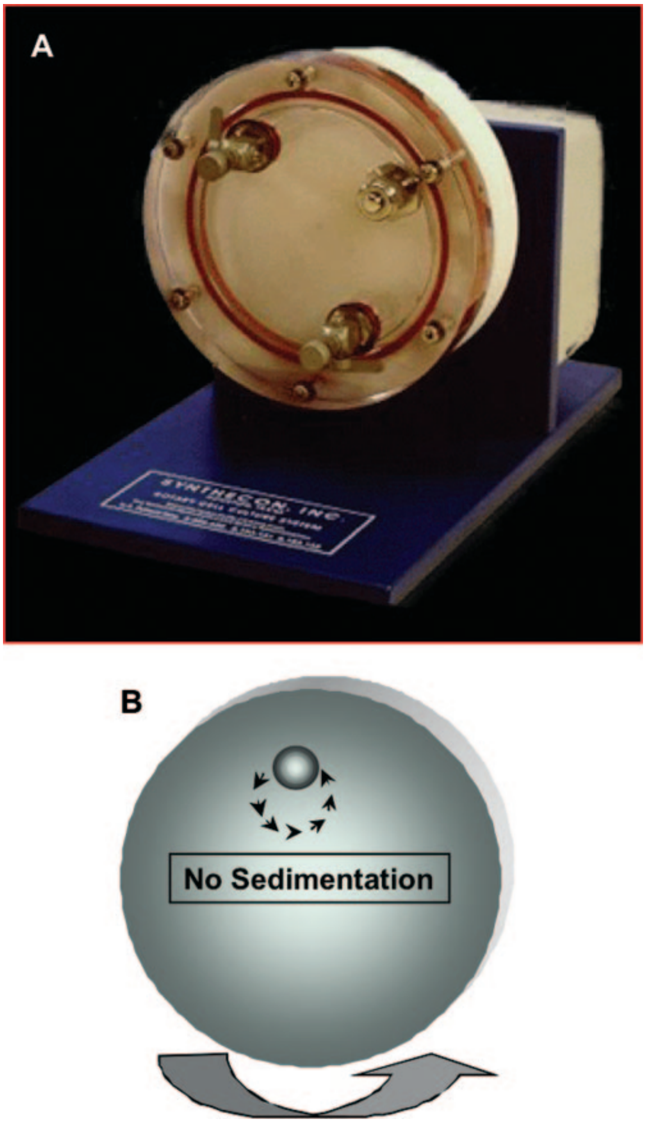

FIG. 1. The RWV bioreactor. (A) The cylindrical culture vessel is completely filled with culture medium through ports on the face of the vessel and operates by rotating around a central axis. Cultures are aerated through a hydrophobic membrane that covers the back of the cylinder. (B) Cells cultured in the RWV are maintained in a gentle fluid orbit (i.e., suspension culture).

constant gas exchange during growth. In addition, the low-fluid-shear growth environment achieved through optimized suspension culture in the RWV provides growth cues which are relevant to those encountered in the placenta and other low-fluid-shear areas of the body such as between the brush border microvilli of epithelial cells (1, 5, 6, 15). The latter in vivo environment is relevant to studies involving enteric pathogens, such as *Salmonella*, since it is likely to occupy this niche between the microvilli of epithelial cells in the intestine and other tissues during the natural course of infection.

Studies of bacterial cultures grown in the RWV have indicated multiple phenotypic and gene expression changes in response to the low-fluid-shear environment of the bioreactor (9, 17, 19, 20, 31, 32). Previous investigations using the enteric bacterial pathogen *S. enterica* serovar Typhimurium indicated that the low-fluid-shear culture environment in the RWV serves as a global regulator of gene expression, stress resistance, and physiology and increases the virulence of this organism, as compared to increased fluid shear conditions (19, 31, 32). Physiological changes resulting from low-fluid-shear growth in the RWV have also been documented in other bacteria, including *Escherichia coli* (8, 9, 17), *Bacillus brevis* (10), and *Streptomyces clavuligerus* (11). Interestingly, Fang et al. observed that the addition of a large particle (a 1/8-in. Teflon bead) into the RWV bioreactor created enough shear to disrupt the low-fluid-shear environment during bacterial culture (8). However, the fluid shear imparted to the culture was not quantified and the effect of smaller particles was not evaluated. This finding prompted us to add beads of different sizes to the RWV to increase the levels of fluid shear force within the bioreactor to quantified, physiological levels and study the corresponding effects on bacterial cells.

Early modeling work by Gao et al. on fluid shear in the RWV considered the effect of adding a spherical microcarrier particle with a radius small enough not to significantly disrupt the solid body rotation of the fluid in the bioreactor (13). Gao's studies on the fluid dynamics in the RWV were among the first to demonstrate that culture conditions within the bioreactor are characterized by a low-fluid-shear environment (13). However, the use of a larger particle in the RWV, like that used by Fang et al., as well as by us in the present study, would indeed disrupt the fluid's solid body rotation. This disruption would introduce a velocity gradient and increased levels of shear stress in the fluid—thus requiring development of a new model. Therefore, based on the observations of Fang et al. and the early modeling work of Gao et al., we developed a new quantitative model of the RWV containing a single bead appropriate for the evaluation of fluid shear forces on planktonic bacterial cells. By using beads of different sizes in the RWV, the fluid shear imparted to the culture can be adjusted to levels that the bacteria may experience during the infection process.

We evaluated the stress response and changes in gene expression of planktonic cultures of *S. enterica* serovar Typhimurium in response to incremental changes in quantified physiological fluid shear using this model. When exposed to progressively higher fluid shear levels, cultures of *S. enterica* serovar Typhimurium displayed corresponding progressive changes in both phenotypic and gene expression characteristics. This study suggests the potential of a fluid shear-based mechanism in the pathogen *S. enterica* serovar Typhimurium, which could influence microbial response during the infection process and may lead to the discovery of new targets for vaccine and therapeutic development.

MATERIALS AND METHODS

Mathematical modeling. The equilibrium position of the bead in the RWV bioreactor was measured during each experiment, and a representative value for the radius and angular position was used to construct the models of the bioreactor using a commercial software package, FEMLAB 3.1 (Comsol, Burlington, MA). The FEMLAB software was used to solve the Navier-Stokes equations for fluid flow throughout the bioreactor volume. For all studies, the rotation rate of the bioreactor was 25 rpm. Polypropylene beads used in this study were obtained from Baltec, Inc., Los Angeles, CA.

In order to complete the model formulation, it was necessary to apply appropriate boundary conditions. The outside edges and back face of the bioreactor were given the same angular rate of rotation of 25 rpm, and the front face was modeled as a plane of symmetry so that it was only necessary to model half of the volume. In order to solve the problem, the model was divided into a grid, or mesh, of smaller elements. A convergence study that monitored the peak fluid shear stress was used to determine the optimal number of elements for each model. For the 1/8-in. bead, the mesh contained a total of 10,230 elements. For the 3/32-in. bead, the mesh was generated in the same manner and consisted of 11,218 elements. The bacteria are extremely small compared to the size of the beads and, consequently, the model assumes that they do not substantially disrupt the fluid flow. For the purposes of this model, the fluid was assumed to be Newtonian with a density of 1,000 kg/m^3 and a viscosity of 0.001 kg/(m · s). The steady-state Navier-Stokes equations were used to determine the velocity field for each model. The velocity increased radially, except for the region near the spherical bead.

It should be noted that this model does not include two fluid dynamic effects that have been studied previously. First, we have neglected the transient motion of the particle because the particle reaches its steady-state position within 1 to 2 min in the RWV (21). Consequently, the effect of the transient motion on the long-term (10 h) behavior of *Salmonella* should be negligible. Second, the bead oscillates slightly about its steady-state position (2). Our model has neglected this motion because our calculations indicate that it does not substantially affect the magnitude of the fluid shear stress, although it may enable the bead to affect a greater area of the flow field and as a result influence more cells than what is predicted. In addition, it should be noted that, over the course of the 10-h experiment, the equilibrium position of the bead moved radially outward a small, but noticeable, amount. This drift indicates that cell proliferation increased the viscosity of the fluid throughout the experiment, thereby causing a slight shift in the equilibrium position of the bead. Consequently, the shear stress would be expected to increase slightly relative to the constants used for this model, but these parameters provide accurate predictions of the changes in fluid shear stress resulting from the introduction of a lighter-than-water bead into the bioreactor.

Bacterial cultures. All studies were performed with wild-type *S. enterica* serovar Typhimurium strain χ3339, an animal-passaged isolate of SL1344 (14) grown in Lennox broth (LB) as previously described (19). Briefly, static overnight cultures grown at 37°C were diluted 1:200 in fresh medium and introduced into the RWV bioreactor (50-ml volume) (Synthecon, Inc., Houston, TX) such that the bioreactor was completely filled with culture medium and no air bubbles were present. All incubations in the RWV were done at 37°C with a rotation rate of 25 rpm. The RWV cultures were harvested after 10 h of growth, which corresponded to the mid-to-late logarithmic phase of growth (19, 31).

Environmental stress assays. Strains grown in the RWV bioreactors were immediately subjected to the particular stress being tested. The stresses assayed in this study, acid and thermal, were chosen because we have previously shown that the low-fluid-shear culture environment in the RWV (i.e., without beads) modifies the *Salmonella* stress response to these conditions (19, 31). Cultures were removed from the RWV and placed into a static culture at 37°C for immediate analysis. For acid stress, the pH of the harvested cultures was lowered to 3.5 by the addition of an amount of concentrated citrate buffer that had been previously determined to give this pH value. The pH level during the assay was monitored with pH strips and then confirmed with a pH electrode at the end of the assay. The acid stress assay was performed statically at room temperature for 30 min. For thermal stress, the harvested cultures were immediately transferred to heating blocks set at 55°C and assayed for 30 min. For both the stress assays, samples were removed at time zero (before the addition of stress) and at various time points thereafter and plated on LB agar to determine the numbers of viable CFU. Percent survival was calculated as the number of CFU at each time point divided by the number of CFU at time zero. At least three independent trials were performed for each stress experiment. The means and standard deviation values are given in Table 1.

Genetic assays. The promoter region upstream of the *S. enterica* serovar Typhimurium gene *rtsA* was fused to the promoterless *lacZ* reporter gene on plasmid pQlacZ1, as previously described, to create plasmid pJWT66 (30). The Km^r gene from plasmid pCR4-TOPO (Invitrogen) was PCR amplified and cloned onto pQlacZ1 at the EcoRI site (such that the Km^r gene promoter drives *lacZ* expression constitutively) to create plasmid pJWT67. Plasmids pJWT66 and pJWT67 were transferred to the *S. enterica* serovar Typhimurium strain χ3339 via conjugation, and the *lacZ* activity of the resulting strains was determined as described previously (18) after growth in the RWV without any bead and with the 1/8-in. bead for 10 h in LB medium (corresponding to the late-log phase) as described above. The data are from three independent experiments, each performed with triplicate samples. Standard errors of the means are given.

TABLE 1. Thermal and acid stress responses of *S. enterica* serovar Typhimurium cultured in the RWV with and without addition of a 3/32- or 1/8-in.-diameter polypropylene bead[a]

Condition	Maximum shear (dynes/cm^2)	% Survival	
		Thermal stress	Acid stress
No bead	<0.01	7.5 ± 1.0	58.1 ± 2.5
3/32-in. bead	5.2	4.9 ± 0.6	51.6 ± 1.5
1/8-in. bead	7.8	2.9 ± 1.0	33.3 ± 4.2

[a] Cultures were removed from the RWV and immediately incubated at either 55°C for 30 min or at pH 3.5 for 30 min.

RESULTS

Computational modeling of bead addition to the RWV. The ultimate goal of this study was to model physiological fluid shear forces created by bead addition to the RWV and to examine the subsequent effects on planktonically grown bacterial cells. To accomplish this goal, the positions of a 3/32-in. bead (2.381 mm) and a 1/8-in. bead (3.175 mm) were observed in separate trials during RWV operation. The distance of the bead from the center and angular position within the RWV were recorded through in situ imaging. The observed steady-state position for the beads used in our model was 35.5 mm from the center and 4.5 degrees below horizontal for the 1/8-in.-diameter bead. For the 3/32-in. bead, the equilibrium radius was 25.0 mm and it came to rest 3.0 degrees below the horizontal (Fig. 2). These values were used to construct the models in FEMLAB 3.1 software (Comsol, Burlington, MA), incorporating the fluid shear effects of the bead with the front and rear faces of the bioreactor.

The results from our model clearly indicated that the maximum fluid shear within the reactor was developed at the surface of the bead where the greatest velocity gradients were calculated (Fig. 3). Calculation of the fluid shear indicated that the maximum shear stress of 7.8 dynes/cm^2 occurred at the outer edge of the 1/8-in.-diameter bead closest to the outer surface of the RWV (Fig. 4). The maximum shear stress at the surface of the 3/32-in.-diameter bead was 5.2 dynes/cm^2. For comparison, the fluid shear experienced by the bacteria on their cell surface without a bead in the bioreactor was estimated to be less than 0.01 dyne/cm^2. While the fluid shear stress decreased exponentially with distance from the bead in both cases, the shear stress in the fluid surrounding the 1/8-in. bead was consistently 50% greater than that in the fluid immediately surrounding the 3/32-in. bead. Thus, the addition of a 1/8- or 3/32-in.-diameter bead exposed the bacterial culture to fluid shear within a range of 7.8 to 0.01 dyne/cm^2, or 5.2 to 0.01 dyne/cm^2, respectively. As previously mentioned, these ranges are relevant to those encountered by *Salmonella* and other bacterial pathogens in an infected host (1, 5–7, 15, 24).

The effect of incremental fluid shear changes on cultures of *S. enterica* serovar Typhimurium. To demonstrate the utility of our model system to elicit cellular responses, we grew *S. enterica* serovar Typhimurium in the bioreactor with either a 1/8-in. bead, a 3/32-in. bead, or no bead, respectively. No difference in bacterial growth or oxygen concentrations was observed in the RWV in either the presence or absence of beads immediately upon extraction of cells from the bioreactor (data not shown), reinforcing that observed differences were the

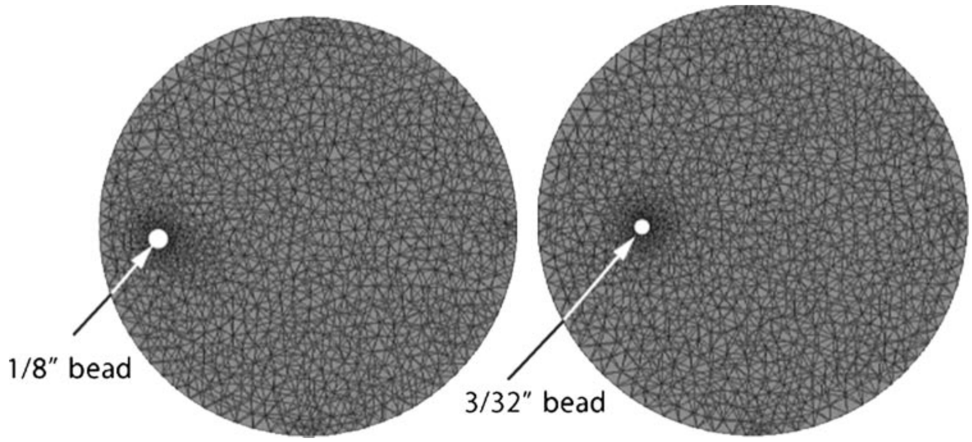

FIG. 2. Model of the fluid in the bioreactor moving past a stationary polypropylene bead. (The 1/8- and 3/32-in. beads are shown in this model.) The equilibrium position of each bead was determined experimentally and used to build the model geometry. In order to solve the problem, the model was divided into a grid, or mesh, of smaller elements. For the 1/8-in. bead, the model consisted of 10,230 elements. For the 3/32-in. bead, the model consisted of 11,218 elements.

result of fluid shear rather than other physical conditions, such as mass diffusion. *S. enterica* serovar Typhimurium cultures displayed a decreasing percentage of survival of both acid stress and thermal stress that was dependent on the level of fluid shear imparted from the bead (Table 1). Specifically, higher fluid shear levels resulted in decreased survival of *Salmonella* in both acid and thermal stresses. Interestingly, the addition of both 1/8- and 3/32-in. beads simultaneously or the addition of multiple (five) 1/8-in. beads simultaneously did not significantly decrease either the thermal or acid stress percent survival of *S. enterica* serovar Typhimurium cultures as compared to those cultured with a single 1/8-in. bead (data not shown).

We have previously shown that the low-fluid-shear growth environment of the RWV (no bead addition) altered *Salmonella* gene expression (32). Therefore, we evaluated the relationship between *Salmonella* gene expression and increasing fluid shear based on addition of a 1/8-in. bead to the bioreactor

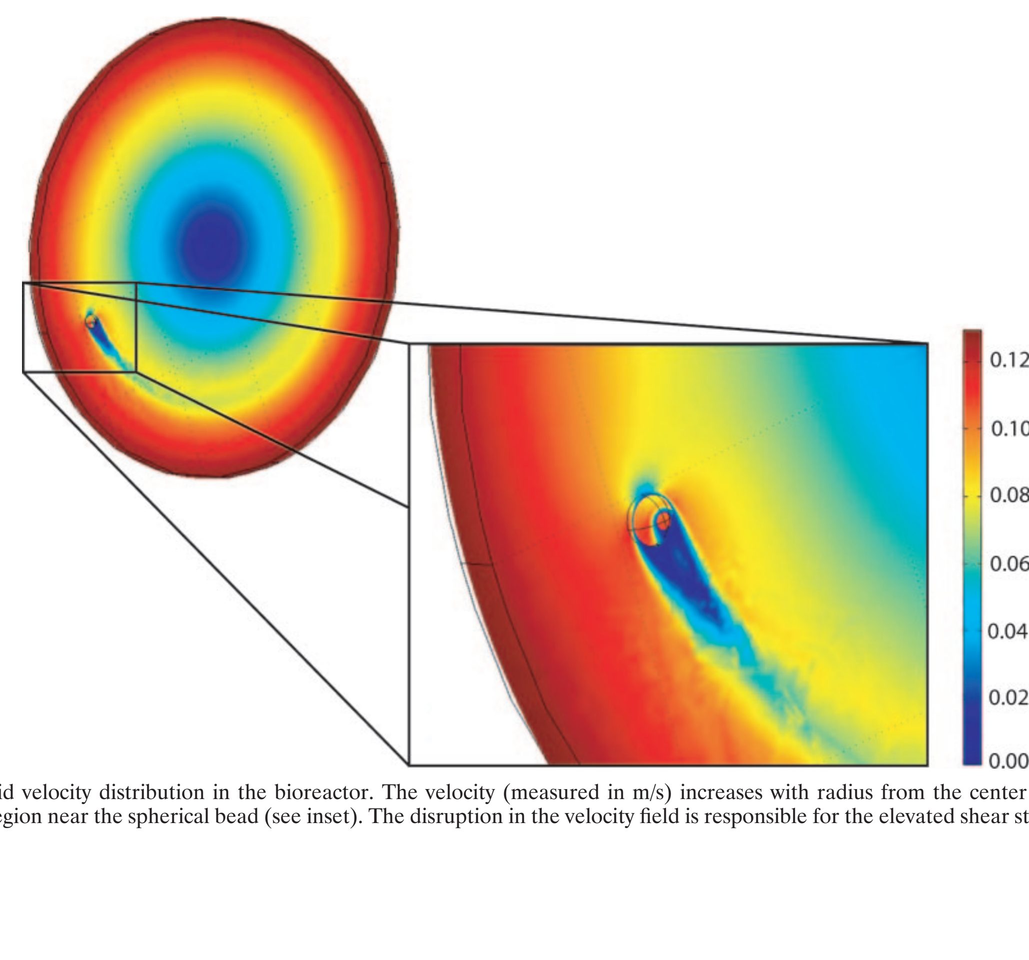

FIG. 3. Fluid velocity distribution in the bioreactor. The velocity (measured in m/s) increases with radius from the center of the bioreactor except in the region near the spherical bead (see inset). The disruption in the velocity field is responsible for the elevated shear stresses in the fluid.

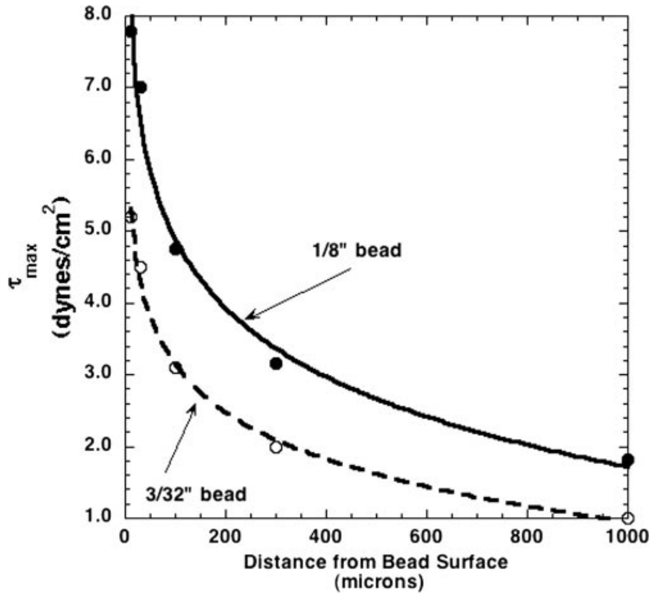

FIG. 4. Maximum shear stress in the fluid surrounding the stationary bead as a function of distance from the surface of the bead. The shear stress decreases exponentially with distance in both cases, but the shear stress in the fluid surrounding the 1/8-in. bead is consistently 50% greater than that in the fluid surrounding the 3/32-in. bead.

(Fig. 5). We constructed a *lacZ* transcriptional fusion to the promoter from gene *rtsA* (located in a cluster of *S. enterica* serovar Typhimurium genes previously shown to be differentially regulated in response to low fluid shear) (32) and assayed expression of this gene in *S. enterica* serovar Typhimurium in the RWV with and without the addition of a 1/8-in. bead (Fig. 5). Expression of the *rtsA* promoter was related to bead-induced shear differences in the bioreactor, as increased transcription levels were observed in response to addition of the bead. A control *lacZ* fusion to the promoter from the Km^r gene did not show evidence of a fluid shear-related response.

DISCUSSION

Microorganisms have developed sophisticated responses to a wide variety of environmental conditions (12, 23), including fluid shear (3, 19, 25, 26, 30, 31, 32). Understanding the mechanism by which the mechanical stimulus of fluid shear changes microbial characteristics has the potential to provide insight into many aspects of microbial properties, including those important for the host-pathogen interaction. Our computational model and bioreactor system provides accurate estimates of adjustable fluid shear levels in planktonic, growing cultures with sufficient cell numbers to perform multiple genotypic and phenotypic assays.

In this study, the addition of a bead into the RWV did not affect the growth curves or oxygen utilization of bacterial cells in the vessel, suggesting changes in microbial characteristics due to mass transfer are unlikely. Consequently, the most likely cause of the change in the biological response of *Salmonella* observed in the presence or absence of beads in the bioreactor is the difference in fluid shear stress. While the fluid shear within the vessel is not homogeneous, the range with the addition of either bead tested in our system is physiologically relevant, and microbial characteristics associated with this fluid shear range can be directly compared to other ranges. Because the fluid velocity is relatively slow within the bioreactor, there are no effects of turbulence and the distribution of fluid shear stress is predictable. It should also be noted that the fluid shear microenvironment within the infected host is also necessarily heterogeneous. While the degree of heterogeneity in vivo is

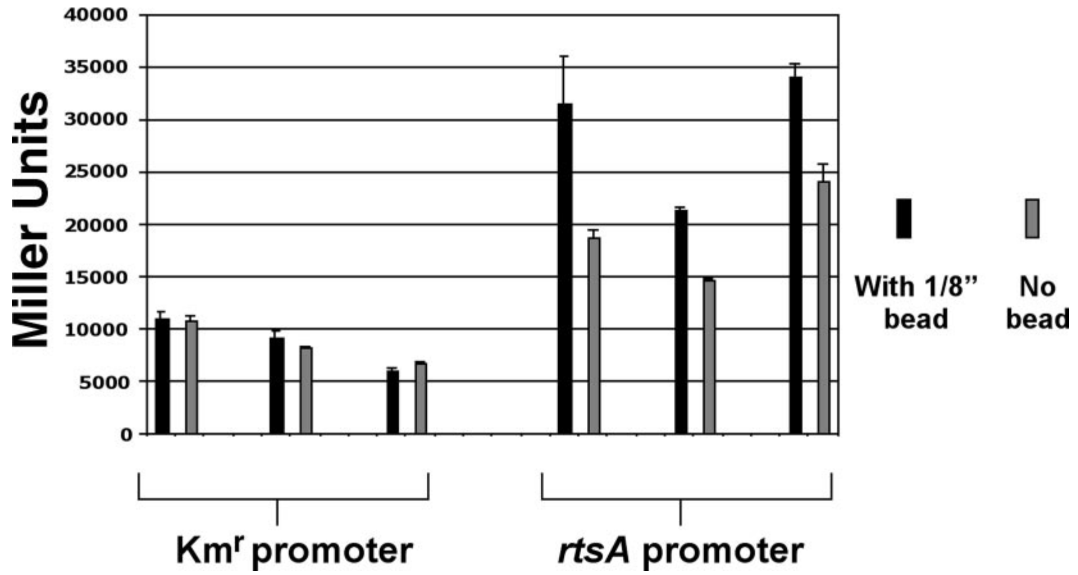

FIG. 5. Measurement of *S. enterica* serovar Typhimurium gene expression in the RWV with and without the addition of a 1/8-in. bead. The promoter for *S. enterica* serovar Typhimurium gene *rtsA* was fused to a *lacZ* reporter, and its activity was measured during growth of the fusion strain in the RWV with and without the 1/8-in. bead. The gene *rtsA* is located in a cluster of shear-regulated genes in the *S. enterica* serovar Typhimurium genome. A Km^r gene promoter predicted to be unaffected by changes in shear was fused to *lacZ* and used as a control. The data from three independent trials each performed with triplicate samples are shown for each strain. Standard errors of the means are given.

difficult to quantify, it may be an important aspect of the overall population response. Previous work in our lab has shown that low fluid shear globally alters the virulence, stress resistance, and gene expression profiles of *S. enterica* serovar Typhimurium (19, 20, 31, 32). In this study, the addition of beads to the RWV provided a range of fluid shear levels predicted to be relevant to those encountered by *Salmonella* and other pathogens during infection of the host intestinal tract and bloodstream (1, 5, 6, 15, 24). The potential of an enteric pathogen like *Salmonella* to alter its gene expression and phenotypic and virulence characteristics in response to the fluid shear levels encountered during infection of the host presents the exciting possibility to enhance our knowledge of the infection process and develop targets for therapeutics and vaccines. Another key advantage of our model and bioreactor system is its use of planktonic bacteria, which would avoid a mechanical force on the cell membrane caused by adherence to a solid surface (22, 29), thus potentially providing a clearer picture of the effect of fluid shear alone on the cellular response.

To demonstrate the application of this model, we subjected cultures of *S. enterica* serovar Typhimurium to three physiologically relevant fluid shear ranges during growth through the mid-late logarithmic phase in the RVW (19). In response to both thermal and acid stresses, *S. enterica* serovar Typhimurium demonstrated progressively lower survival rates as the fluid shear was increased. Earlier studies indicated higher thermal and acid survival of *Salmonella* in response to growth at lower, though unquantified fluid shear in the RWV (19, 31). This study confirms a direct relationship between the stress response of *Salmonella* to acid and thermal stresses and the diameter of the bead, indicating a relationship between the stress response and incrementally increasing fluid shear based upon our mathematical modeling. In addition, the expression of the *rtsA* promoter in response to increased fluid shear is also consistent with our previously published work using the RWV (32). Interestingly, the *rtsA* gene encodes a regulatory protein that has been implicated in *S. enterica* serovar Typhimurium invasion of the small intestine (7a). Accordingly, our findings indicate that changes in fluid shear levels correlate to a molecular change (i.e., gene expression) in a promoter predicted to be affected by fluid shear. Moreover, in support of observations by Fang et al. (8), we found that the addition of multiple beads to our system did not result in further changes in acid stress response. This observation suggests that a single 1/8-in. bead provides substantial disruption in the low-fluid-shear environment, and a limit or "threshold" on the fluid shear effect may exist. Likewise, we observed a similar "threshold" effect for *Salmonella* for the thermal stress response. The potential of environmental responses that occur below a threshold reinforces the potential that these changes could benefit a pathogen in a low-fluid-shear environment.

The development of a mathematical model capable of defining fluid shear ranges imparted to a microbial culture has the potential to provide a mechanism for understanding the relationship of microorganisms to the mechanical fluid forces they encounter during their natural life cycles. This study provides a novel model system to evaluate microorganisms at quantifiable ranges of physiologically relevant fluid shear levels. To our knowledge, this is the first report to provide evidence that incremental changes in fluid shear can cause corresponding changes in biological response in the bacterium *S. enterica* serovar Typhimurium and may possibly translate to other growing cultures of planktonic bacteria. By applying this model to *S. enterica* serovar Typhimurium and other pathogens, entire classes of genes and proteins involved in cellular interactions may be discovered that have not previously been identified during growth under conventional culture conditions, providing new targets for vaccine and therapeutic development.

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